ON THE ORIGIN AND ACTION OF HEMOLYTIC COMPLEMENT.*

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The rôle of complement in bacteriolytic processes is familiar. Recent investigations emphasize the importance of the complement of the blood in the parenteral digestion of proteins and in infections. Thus Friedberger and his associates[1] produced anaphylatoxic substances from bacterial and other proteins by means of the action of complement. Variations in the complement content of the blood during disease have been noted by Moro,[2] who concluded that a capacity for the ready formation of complement was of prognostic value. I have found[3] a decrease in hemolytic complement before the crisis and an increase at the time of crisis in pneumonia. Lüdke and others[4] found a decrease in complement in chronic suppurative processes. Eliasberg[5] found the blood of leprous patients poor in complement. In view of the possibility of raising the complement content of the blood for therapeutic purposes, it is rather surprising that there is so little unity of opinion either as to the origin of complement or as to the nature of the chemical processes with which it is concerned.

THE ORIGIN OF COMPLEMENT.

Since Buchner and Metchnikoff advanced the idea that complement was derived from the leukocytes, that idea has been the prevalent one, although there has been a great deal of experimental evidence both for and against it. Other locations which have been considered as a source of complement are the pancreas and liver.

1. *The leukocytes.*—The following points are advanced as evidence for and against the leukocytes as a source of complement:

a) Complement does not exist free in the plasma as it is removed from the body (Gengou[6]). The most recent confirmation of this evidence is by Gurd.[7] Contrary

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[1] *Ztschr. f. Immunit.*, 1911, 2, p. 471.

[2] *Über das Verhalten hemolytische Serumstoffe beim gesunden und kranken Kind*, Wiesbaden, 1908.

[3] *Jour. Infect. Dis.*, 1912, 10, p. 383.

[4] Quoted by Sachs, *Handbuch der Technik und Methode der Imm. Forsch.*, Gustav Fischer, Jena, 1909

[5] *Deutsch. med. Wchnschr.*, 1901, 37, p. 302.

[6] *Ann. de l'Inst. Past.*, 1901, 15, p. 232.

[7] *Jour. Infect. Dis.*, 1912, 11, p. 225.

evidence is brought forward by a number of investigators. The work of Addis[1] seems especially convincing.

b) Serum left in contact with leukocytes (blood-clot) increases in complement content (Walker,[2] Smith,[3] Longcope,[4] and Gurd[5]). Of those whose work tends to disprove this evidence Addis[6] may be again cited.

c) Extract of organs containing many leukocytes acts as complement (Tarassevitch[7]). This is denied by Korshun and Morgenroth,[8] who were able to obtain a hemolysin from organ extracts, but this hemolysin did not act as complement as it was inhibited in its action by amboceptor.

2. *The pancreas.*—Sweet[9] concluded that after extirpation of the pancreas in dogs, the complement content fell. This conclusion, however, was based on observations made on the serum of one animal. This serum was removed after death and the complement content was not directly estimated, but the total lytic power was found to be decreased. On account of the fact that no diminution in amboceptor could be ascertained, the decrease in total lytic power was thought due to a decrease in complement.

3. *The liver.*—Müller[10] obtained an increase in both amboceptor and complement in the blood by means of injections of iodipin and attributes the action of this drug to an influence on the liver. His reason for regarding the liver as the source of complement is that serum perfused through the liver became stronger in complement. Recently Gay and Rusk[11] have been unable to obtain any increase of antibody production with iodipin.

In view of these conflicting ideas, it was thought desirable to make an investigation of the following points: (1) The presence of complement in plasma; (2) the influence of the blood-clot and its components on the complement content of serum left in contact with it; (3) a comparison of the complement in the fluid from leukocytic exudates with that of the blood serum.

In the complement estimations the following plan was used. An antisheep rabbit serum inactivated for one-half hour at 56° C. was used as an amboceptor. This was first titrated to determine the minimum dose required to cause lysis of a 5 per cent suspension of washed sheep erythrocytes with one-tenth cubic centimeter of 1:10 dilution of normal guinea-pig serum. The total volume of the mixture was always one cubic centimeter. This minimum dose was considered as one unit of amboceptor. A comparison was then made of the amounts of test and control serums required to give the same amount of hemolysis using 10 units of amboceptor.

[1] *Jour. Infect. Dis.*, 1912, 10, p. 200.
[2] *Jour. Hyg.*, 1903, 3, p. 52.
[3] *Proc. Royal Soc.*, 1906, 79, p. 378.
[4] *Med. Bull. Univ. of Penna.*, 1902.
[5] *Op. cit.*
[6] *Op. cit.*
[7] *Ann. de l'Inst. Past.*, 1902, 16, p. 127.
[8] *Berl. klin. Wchnschr.*, 1902, 5, p. 870.
[9] *Jour. Med. Res.*, 1903, 10, p. 255.
[10] *Centralbl. f. Bakt.*, 1911, 57, p. 577.
[11] *University of California Publications*, 1912, 2, p. 73.

Those tubes in which there was but a moderate or beginning hemolysis were compared.

1. *The amount of complement in paraffin plasma.*—An aspirator with a large needle was filled with liquid paraffin and the paraffin partially expelled. The needle was then inserted into the heart of a guinea-pig and about five cubic centimeters of blood aspirated. The blood was immediately centrifuged in a centrifuge tube coated with liquid paraffin and containing a small amount of paraffin in the bottom. The plasma was then pipetted off and placed in a small test tube. The plasma coagulated at once. A part of the serum was taken from the fibrin and one-half of it used as complement in mixtures which had been made up and were in readiness excepting for the addition of complement. The other half of the serum was incubated for one-half hour as was also the serum fibrin mixture. Two sets of tubes were then arranged similar to the first set, excepting that in the one incubated serum was used as complement, and in the other serum incubated with fibrin. After incubation for two hours all three sets of tubes were read and put on ice until morning and then read again. As is shown in the following table, the fractions of serum treated in the different ways all contained the same amounts of complement.

TABLE 1.

HEMOLYSIS WITH SERUM TREATED IN VARIOUS WAYS.

Treatment of Serum	Per Cent of Hemolysis with Varying Amounts of Serum Diluted 1:100				
	0.05 c.c.	0.1 c.c.	0.2 c.c.	0.3 c.c.	0.4 c.c.
Serum removed from plasma at once	0	5	10	25	40
Serum incubated with fibrin one-half hour	0	5	10	20	40
Serum incubated alone one-half hour	0	5	10	20	50

The difference in the results of this experiment and in those of Gurd[1] may possibly be explained by the fact that ether was used in Gurd's experiments, although an incubation for a short time would serve to get rid of at least part of the ether, yet Guggenheimer[2] has shown that ether has an inhibiting effect on the action of complement.

[1] *Op. cit.* [2] *Ztschr. f. Immunit.*, 1911, 11, p. 30.

2. *The influence of the blood-clot and its components upon the complement content of the serum left in contact with it.*—A part of the serum from a few cubic centimeters of guinea-pig blood was obtained by centrifugation immediately after removal and placed on ice. The remainder of the serum was left with the clot in the ice-box for 16 hours. The two parts of the serum were then compared as to the complement content in the way described above in experiment 1. The result was as follows:

TABLE 2.

HEMOLYSIS WITH SERUM SEPARATED AT ONCE AND LEFT WITH CLOT.

Serum	Per Cent Hemolysis with Varying Amounts of Serum Diluted 1:100		
	0.2 c.c	0.4 c.c.	0.6 c.c.
Left with clot 16 hours	30	60	90
Separated at once	40	70	90

There is no difference in the complement content of the two different fractions of the serum which may not be explained by error.

On the supposition that the complement content of serum might be increased by contact with one or more of the components of the clot the following experiment was made. Blood was obtained as in the first experiment and centrifuged in liquid paraffin. As blood so treated separates into a layer of erythrocytes at the bottom, a layer of leukocytes covering this, and above this the plasma, it was possible to obtain a quantity of erythrocytes, leukocytes, and fibrin. Serum left in contact with each of these for 16 hours in the ice-box was compared with serum removed at once.

Table 3 shows that in experiments 1 and 2 there is a diminution of complement content in serum left in contact with white cells and fibrin, whereas in experiment 3 the serum left in contact with the leukocytes is strongest in complement; in experiment 4 the highest complement content is found in that serum which was left in contact with the fibrin, while in experiment 6 the highest content is in the serum incubated with erythrocytes.

It is very unlikely that any of the differences in the tables are

TABLE 3.

HEMOLYSIS WITH SERUM TREATED VARIOUSLY. THE FIGURES GIVE THE PERCENTAGE OF HEMOLYSIS WITH VARYING AMOUNTS OF SERUM DILUTED 1:100.

Treatment of Serum	Experiment 1 (Serum Incubated with Blood Elements, etc., for 16 Hours)			Experiment 2 (Serum Incubated with Blood Elements, etc., for 16 Hours)			Experiment 3 (Serum Incubated with Blood Elements, etc., for 16 Hours)				Experiment 4 (Serum Incubated Two Hours with Blood Elements, etc.)					Experiment 5 (Serum as in Experiment 4)		
	0.2 c.c.	0.3 c.c.	0.4 c.c.	0.2 c.c.	0.3 c.c.	0.4 c.c.	0.1 c.c.	0.2 c.c.	0.3 c.c.	0.4 c.c.	0.2 c.c.	0.3 c.c.	0.4 c.c.	0.5 c.c.	1.0 c.c.	0.2 c.c.	0.3 c.c.	0.4 c.c.
Serum removed at once	40	80	90	10	20	25	0	10	20	25	10	15	25	30	50	15	20	25
Serum left with clot	40	80	90	10	25	30	0	10	20	25	..	..	..	..	..	15	20	25
Serum left with fibrin	40	80	90	10	25	30	0	10	25	35	10	20	30	40	60	15	20	25
Serum left with fibrin and leukocytes	20	60	80	10	20	25	0	10	20	25	..	..	..	..	..	15	20	25
Serum left with leukocytes	40	90	90	10	20	25	0	10	25	40	10	15	25	30	50	15	15	20
Serum left with leukocytes	55	90	90	10	25	30	0	10	20	25	10	20	30	35	50	..	25	30
Mixture of equal parts of all sera	40	90	90	10	20	25	0	10	20	25	..	..	..	..	80	15	20	25

outside of the limits of unavoidable error. The experiments therefore fail to indicate any connection between complement formation and the blood-clot or its components. Much of the work which has been taken to indicate that leukocytes are the source of complement has been done on bacteriolytic complement. Bacteriolytic experiments are, as compared with hemolytic, less accurate, and although the possibility of the difference in result being due to a difference in the place of formation of the two complements cannot be denied, the more likely explanation of the difference in the result is in the inaccuracy of bacteriolytic work.

3. *Comparison of the complement content of leukocytic exudates with that of the blood serum.*—The independence of complement content with respect to the number of leukocytes or leukocytic disintegration is shown by a comparison of the amount of complement of fluids rich and poor in leukocytes with that of the serum.

A dog was injected intrapleurally with aleuronat suspension and the following fluids compared: (1) that from the centrifugated leukocytic exudate; (2) the fluid obtained from the centrifugated leukocytes by pressure; (3) the cerebrospinal fluid almost free from leukocytes; (4) the blood serum. It required one and one-half times as much exudate fluid to produce any given percentage of hemolysis as was the case with serum, three times as much leukocytic extract, and four times as much cerebrospinal fluid. The blood serum of the animal might much more reasonably be considered the source of the complement in the various fluids examined than the leukocytes.

Inasmuch as no indication that the leukocytes formed complement could be found, an attempt was made to obtain complement from other parts of the body as follows: A dog was etherized and the femoral vein isolated on one side and the artery on the other. A cannula connected with a reservoir of normal salt solution was inserted into the vein and salt solution allowed to flow in as the dog was bled from the opposite femoral artery. After the liquid from the artery became colorless the various organs were removed from the body and where possible, as in the liver, spleen, and kidney, perfused again with salt solution. Fluids were obtained from the organs by simple pressure, by grinding with salt solution with and

without sand, and by extraction with ether and alcohol. The experiment was repeated a number of times with guinea-pigs and in no instance was a fluid obtained from any of the organs which was capable of acting as complement. In the case of the lipoid extracts some hemolysis with the extract alone was obtainable, but the addition of amboceptor inhibited this hemolysis. It is of especial interest to note that good hemolysis was obtainable with extracts of the stomach mucosa, pancreas, and mucosa of the small intestine. Amboceptor, however, instead of increasing the hemolytic power acted as might be expected as an antiferment. The reaction of these extracts was varied, but in no case was a complement action obtainable. Reference will be made to this hemolysis by proteolytic ferments later. Although it was impossible to obtain from any of the organs a fluid or mixture of fluids which would act as complement, these experiments cannot be taken as indication that none of the organs examined are concerned in complement formation. Accordingly a series of experiments was carried out in which the complement content of the blood of dogs was estimated and then in different cases different organs removed and the effect on the complement content of the blood of this removal noted from day to day until the animal died or until a constant complement content was found day after day. In these complement estimations, the same technic was used as was described in comparison of serum treated in different ways. In this case, however, the serum of guinea-pigs of from 200–300 gms. weight was used as a standard for comparison, as it has been shown by many workers and by a few preliminary experiments that the serum of such guinea-pigs is remarkably constant in complement content. The serum of normal dogs under the conditions of the experiments usually contained about one-sixth the complement activity of normal guinea-pig serum. That is, it took about six times as much dog serum as guinea-pig serum to produce a trace of hemolysis.

Inasmuch as dog blood sometimes contains amboceptor for sheep blood, it was necessary to run controls of the action of the dogs' serum alone without the addition of artificial (rabbit) amboceptor. Wherever the dogs' serum was of itself hemolytic for sheep blood the animals were discarded.

Removal of the pancreas.—The removal of enough of the pancreas to produce a considerable excretion of sugar in the urine was accomplished in sixteen dogs, of these five developed localized infections so that the experiments were worthless. Of the remaining 11 dogs an acidosis was demonstrated in only six while all had a considerable excretion of sugar in the urine and died after a period of from a few days to two weeks.

Of those animals in which an acidosis was present and sugar excreted, the following may be given as an example of the result on the complement content of the blood serum:

Dog 131. Complement content of the serum March 1, one-fifth that of guinea-pig serum. Three hours after pancreatectomy, one-third; two days later, one-fifth; fourth day, one-seventh; 13th day, one-fifth; dead on the 14th day.

Of these animals showing a constant excretion of sugar, but in which no acidosis was demonstrated, the following will serve as an example:

Dog 96. Complement November 22, one-fifth that of guinea-pig serum; about 24 hours after the operation, November 23, one-sixth; November 24, one-fifth; November 25, one-fifth; November 27, two-fifths; December 1, 10 days after the operation, one-third. The dog died on the 11th day.

As an example of the effect of removal of the pancreas and localized peritonitis the following will serve as an example:

Dog 137. April 1, complement one-fifth that of guinea-pig serum; 24 hours after pancreatectomy, one-fifth; April 6, five days after the operation, no complement could be demonstrated. The dog died soon after the last sample of blood was obtained. These experiments indicate that removal of the pancreas in itself has but little effect upon the complement.

Removal of pancreas and duodenum.—Of eight dogs in which the pancreas and duodenum were removed only one showed a change in the complement of the blood. Complement estimations in this dog were as follows:

Dog 104. Before operating, complement one-fifth that of guinea-pig serum. First day after operation, one-half; on succeeding days as follows: one-sixth, one-fifth, one-seventh, one-seventh, one-seventh, one-tenth, one-seventh, one-seventh, one-fifth, one-seventh, one-tenth. On the 14th and 15th days no complement was found and on the 16th day the dog died. No infection was found on postmortem examination.

As an example of the remaining seven dogs which died without change in the complement content of the blood may be given the following:

Dog 108. By mistake no sample before operation was obtained. The first day following operation the complement was one-fifth that of guinea-pig serum. On succeeding days it was two-fifths, one-third, one-tenth, one-third, one-fourth, one-fourth, one-fourth, one-eighth, one-third, one-fifth, one-third. The dog died on the 13th day.

Removal of spleen and pancreas.—Two dogs showed much the same result. Only one will be given in detail:

Dog 105. Before operation, complement one-fourth that of guinea-pig serum. On the days succeeding operation, one-fifth, one-third, one-half, one-tenth, one-tenth, one-tenth, one-seventh, one-tenth, one-third, one-third, one-seventh. The dog died on the 12th day.

Removal of adrenals.—On account of the difficulty in removal of the adrenals only three animals were obtained in which the results can be used. They lived three, four, and 11 hours after operation. In no case was a diminution of complement found. In the dog which lived 11 hours the following estimations were made. Just before operation, complement one-third. Immediately after operation, one-fourth; one hour later, one-fourth; two and one-half hours later, one-fourth; three and one-half hours later, one-third; four and one-half hours later, one-third; 10½ hours later, one-third.

Removal of small intestine.—In each of four dogs approximately six feet of intestine were removed. None of these dogs showed a diminution in complement. One dog lived nearly two months and the complement varied from one-fifth at the beginning to two-fifths a few days before death.

Gastrectomy.—It was possible to remove only the pyloric end of the stomach with but a small part of the cardiac end. In the one dog observed the complement was as follows:

Dog 111. Before operation, complement was one-tenth that of guinea-pig serum. First day, one-tenth; second day, one-tenth; third, two-fifths; fourth, two-fifths; sixth, one-fifth; seventh, one-eighth; eighth, one-half; ninth, three-fifths; 10th, one-fifth; 12th, one-tenth; 13th, one-fifth; 14th, one-tenth; 15th, one-fifth; 16th, one-sixth; 18th, one-sixth; 22d, one-sixth; 60th, one-sixth. The dog was examined after killing with chloroform and the organs found normal.

Thyroidectomy.—The thyroid was removed in three dogs. One of these was kept alive for over a month by feeding calcium lactate, a fourth dog was phloridzinized after removal of the thyroid. The only dog showing a diminution of complement was the phloridzinized dog and in it the complement rose to normal before death. The complement estimations were as follows: Two days following thyroidectomy, the complement was one-sixth; five days after operation, one-sixth. Phloridzin was then begun and two days later complement was one-tenth. On the following day just before the dog was killed the complement was one-fifth or about normal.

Kidney.—No nephrectomies were made but two dogs were injected subcutaneously with bichlorid of mercury. Doses of

one-half and two-tenths gram repectively were given. The first dog died on the following day. The dog receiving two-tenths gm. lived about 55 hours. In neither dog was the complement affected. In the second dog typical bichlorid kidneys were found which on microscopic examination showed extensive destruction of the epithelium and calcium deposit. There was an extensive necrosis of the large intestine with sloughing. The complement estimations were as follows: before the injection, one-sixth; 24 hours later, one-sixth; 48 hours later, one-third. It will be seen that the complement was not diminished by mercuric chlorid poisoning with severe damage to the kidney and colon.

Liver.—On account of the difficulty of removing even a part of the liver, the use of poisons which particularly affect the liver was resorted to. Richards and Howland[1] have shown that if dogs are given chloroform by inhalation for two hours or more, severe damage (usually extensive necrosis) is done to the liver. Accordingly chloroform was given to seven dogs for periods ranging from two to three hours; from $\frac{3}{4}$–2 oz. were given. In two of the dogs the chloroform administration was repeated. In all of these dogs a marked fall in the complement content of the blood was noted. This fall in complement was synchronous with the change in liver, determined by microscopic examination and roughly parallel to the extent of the liver necrosis. An example of complement estimations with chloroform is as follows:

Dog 170. Given chloroform for two hours. Complement before chloroform, one-sixth that of guinea-pig serum. Two hours after administration of chloroform, one-eighth, 22 hours after, one-twelfth; 46 hours after, one-forty-fifth. The dog died a few hours after the last estimation.

In order to be sure that the apparent diminution in complement was not due to the presence of anticomplementary substances in the blood, the following experiment was made. A dog was given chloroform for two hours and by repeated estimations a fall of complement from one-sixth before the chloroform to one-twentieth 96 hours after was ascertained. At this time a quantity of serum was obtained and heated to 56° C. for one-half hour. The minimum amount of normal dog serum required to cause

[1] *Jour. Exper. Med.*, 1909, 11, p. 344.

complete hemolysis with 0.01 c.c. of amboceptor was then found. Then the amounts of heated normal and heated abnormal serum required to inhibit the action of the minimum dose of normal dog serum required to cause complete hemolysis was determined. There was no increase in the inhibiting power of the serum of the chloroformed dogs. An attempt was made to increase the activity of the serum of the chloroformed dog by variation of the reaction of the hemolytic mixtures, using constant quantities of different concentrations of acids and alkalies. In this way concentrations of N/40,000, N/30,000, N/20,000, N/10,000, N/5,000, N/3,500, N/2,000 NaOH and the same strengths of HCl were used. In no case was the complement action increased.

It was suggested by Dr. Wells[1] that the action of hydrazine to be tried in this connection as he has shown that[1] hydrazine is a poison which acts in a peculiarly specific way upon the liver cells.

Dog 150 was given 1.2 gms. of hydrazine sulphate (0.1 gm. per kgm. body weight) subcutaneously. The complement estimations were as follows. Before injection of hydrazine, complement was one-fifth; 24 hours later, one-eighth; 48 hours later, one-tenth; 72 hours later, one-fortieth. The dog died a few hours after the last sample was taken. Extensive necrosis was found microscopically in the liver.

It has been shown by Whipple and Hurwitz[2] that in chloroformed animals a drop in fibrinogen occurs which is proportional to the amount of liver necrosis. Inasmuch as both the fibrinogen and the so-called middle-piece of the complement belong to the globulin fraction of the serum, it was thought desirable to find out whether or not alterations in the middle-piece caused the drop in complement. Accordingly the serum from dog 170 was separated into middle-piece and end-piece by the hydrochloric acid method described by Marks[3] and solutions of end-piece and middle-piece representing each dilution of the original concentration in the serum of 1:10, compared with similar solutions of the middle-piece and end-piece of normal serum. It will be seen from the following table (4) that the middle-piece of the complement-poor serum is equal to the mormal middle-piece in concentration and that the drop in complement is due to a change in the end-piece or albumen fraction and not the globulin fraction as was suspected.

[1] *Ibid.*, 1908, 10, p. 457.

[2] *Ibid.*, 1911, 3, p. 136.

[3] *Ztschr. f. Immunit.*, 1910, 8, p. 508.

TABLE 4.

THE ACTION OF COMPLEMENT-FRACTIONS ON HEMOLYSIS.
Normal end-piece alone hemolyzes with 0.5 c.c., a trace with 0.4 c.c., none with 0.3 c.c.
Complement poor end-piece does not hemolyze.

NORMAL END-PIECE, 0.2 C.C.		NORMAL END-PIECE, 0.3 C.C.	
Liver End-Piece	Normal End-Piece	Liver Mid-Piece	Normal Mid-Piece
0.1 c.c. no hemolysis	0.1 c.c. complete hemolysis	0.1 c.c. complete hemolysis	0.1 c.c. moderate hemolysis
0.2 c.c. no hemolysis	0.2 c.c. complete hemolysis	0.2 c.c. complete hemolysis	0.2 c.c. moderate hemolysis
0.3 c.c. trace	0.3 c.c. complete hemolysis	0.3 c.c. complete hemolysis	0.3 c.c. moderate hemolysis
0.4 c.c. moderate hemolysis	0.4 c.c. complete hemolysis	0.4 c.c. complete hemolysis	0.4 c.c. moderate hemolysis
0.5 c.c. complete hemolysis	0.5 c.c. complete hemolysis	0.5 c.c. complete hemolysis	0.5 c.c. moderate hemolysis

In 1900, Ehrlich and Morgenroth[1] noted that guinea-pig serum loses its hemolytic power for rabbit corpuscles when the animal is poisoned with phosphorus. They were able to restore this power by adding normal guinea-pig serum and concluded that it was the complement that was affected. We have three substances which cause a destruction particularly of the liver tissue and at the same time a diminution of the complement of the blood. This parallelism between complement diminution and liver destruction suggests that either the complement is formed in the liver or that destruction of the liver cells inhibits the formation of complement somewhere else. Inasmuch as these experiments failed to demonstrate any other place of formation, the indications are that it is actually formed in the liver.

THE ACTION OF COMPLEMENT.

It has been a much disputed idea that the complement of the blood is a ferment. In the course of a discussion concerning the ferment nature of complement, Lieberman and Fenyvessy call attention to the fact that none of those who maintain that complement is a ferment advance any ideas as to what chemical processes are concerned in complement action. This investigation has been concerned with (I) the ferment nature of complement; (II) the nature of the hemolytic action of complement and immune body.

I. *The ferment nature of complement.*—The arguments which

[1] *Berl. klin. Wchnschr.*, 1900, 37, p. 683.

have been advanced in favor of the ferment nature of complement are as follows:

1. Complement and immune body do not necessarily act together in definite proportions but an increase in the amount of the one may act as a substitute for a diminution of the other.

2. The reaction velocity of hemolysis depends rather on the concentration than on the absolute amount of complement.

3. Complement is not used up during hemolysis.

4. The action of complement is similar to that of ferment in that it is easily influenced by variations in reactions and salt concentration.

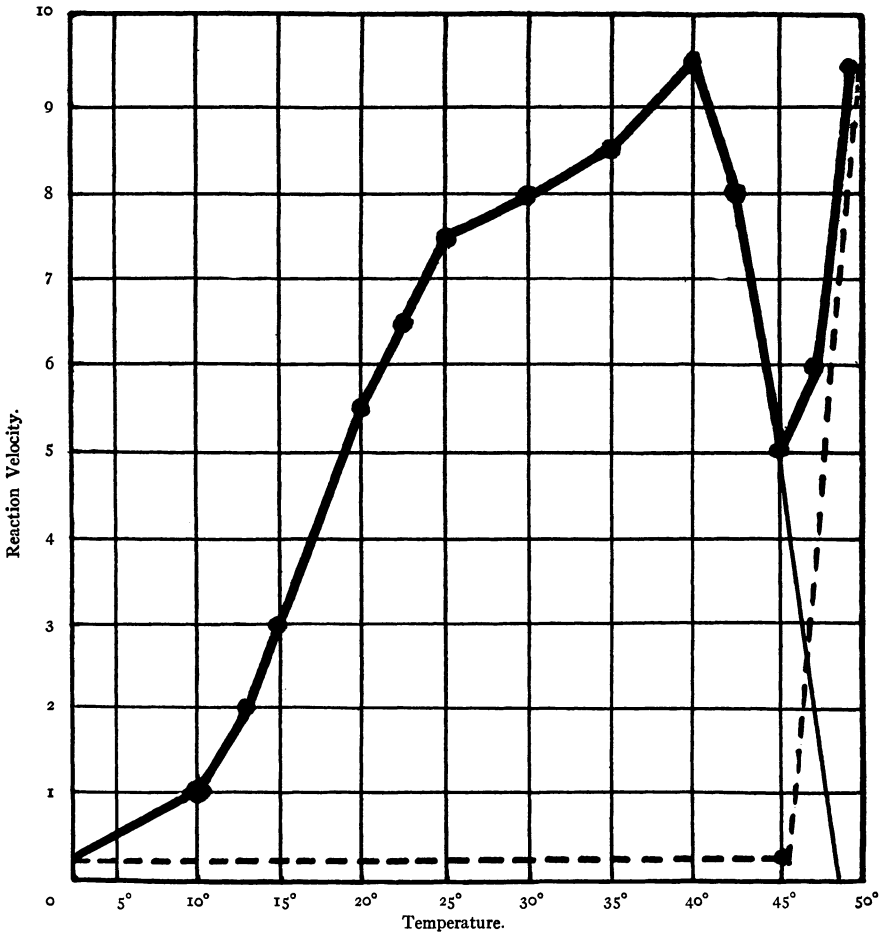
5. The reaction velocity of hemolysis is greatest during the beginning of the process and progressively becomes slower. If this change in reaction velocity is represented by a curve, the curve is very similar in its course to that representing the reaction velocity of trypsin.

It has been shown that instead of the steady increase in velocity of chemical action with increase in temperature, of ordinary chemical reactions, ferment activity shows a marked rise in activity up to a certain optimum and then as the temperature approaches that of the point of destruction of the ferment a sharp fall occurs. Human serum in constant quantity and concentration was allowed to act upon rabbit corpuscles in constant quantity for one hour at different temperatures. The amount of spontaneous hemolysis was estimated by mixtures of heated (56° C. for one-half hour) human serum and corpuscles. The result is expressed in the following curve which resembles closely these representing typical ferment action.

II. *The nature of the chemical processes concerned in hemolysis.*— The close association of complement with proteolytic processes has already been referred to. In the first part of this paper the hemolytic action of extracts of stomach mucosa was noted. The assumption of a proteolysis of the stroma of erythrocytes would readily explain the phenomenon of hemolysis, for with even partial splitting of the complex protein substance of the stroma there would result an increased permeability which would permit of the escape of hemoglobin.

The following experiments were carried out with a view of obtaining direct evidence that proteolysis occurs during hemolysis. A preparation of the stroma of sheep erythrocytes was made accord-

CURVE SHOWING THE INFLUENCE OF TEMPERATURE ON THE REACTION VELOCITY OF COMPLEMENT.



Dotted line indicates action of serum heated to 56° C. for one-half hour.

Heavy continuous line indicates action of normal serum.

At 48° C. the two curves coincide. Fine continuous line indicates reaction velocity of complement action, computed by subtracting percentage of "spontaneous" hemolysis, obtained with heated serum, from percentage caused by unheated serum.

10 per cent hemolysis = a velocity of 1.

ing to Woolridges' method. Two-tenths gram of the dried stroma was powdered in a mortar and suspended in 20 c.c. of normal salt

solution. This suspension was divided into two portions of 10 c.c. each. These two portions were labeled No. I and No. II. To part I were added 0.4 c.c. of amboceptor and one cubic centimeter of complement (guinea-pig serum.) To part II were added 0.4 c.c. of amboceptor and one cubic centimeter of complement which had been heated to 56° C. for one-half hour. The different components were all cooled to nearly freezing point before mixing and as soon as the mixtures were made they were each divided into two equal parts, one of which was immediately centrifuged and five cubic centimeters of the supernatant fluid titrated by the Henriques and Sorenson method for amino-acids. The other half was incubated for two and one-half hours and then treated the same as the first half. The results were as follows:

Mixture I. Ten c.c. of sheep blood stroma suspension, 0.4 c.c. of amboceptor and one cubic centimeter of complement. Formol titration for amino-acids of 5 c.c. of supernatant fluid before incubation, 0.30 c.c. of N/10 NaOH. After incubation, 0.50 c.c. of N/10NaOH. The difference represents the amount of amino-acid formed during the incubation of 0.20 c.c. of N/10 NaOH.

Mixture II. Ten c.c. of sheep blood stroma suspension, 0.4 c.c. of amboceptor, one cubic centimeter of complement. Formol titration of five cubic centimeters of supernatant fluid for amino-acids before incubation, 0.30 c.c. of N/10 normal NaOH. After incubation 0.40 c.c. of N/10 NaOH. The difference represents the amount of amino-acid formed during the incubation of 10 c.c. of N/10 NaOH.

This experiment was repeated a number of times and although the results were similar and tended to show an increased amount of amino-acid in mixtures of stroma, amboceptor, and complement, still the differences were so small that it is questionable if they were outside of the limits of unavoidable error. Accordingly unchanged blood was used in larger quantities and the disturbance of color reactions due to hemoglobin avoided by removing the coagulable albumen in the mixture before estimating the amino-acids. An example of one of these experiments is the following:

Three mixtures were made. No. I contained 15 c.c. of a 100 per cent suspension of washed sheep erythrocytes, 3.5 c.c. of amboceptor (antisheep rabbit serum), and six cubic centimeters of complement (guinea-pig serum). No. II, 15 c.c. of the same blood suspension as No. I and 9.5 c.c. of normal salt solution. No. III, 15 c.c. of normal salt solution, 3.5 c.c. of amboceptor and five cubic centimeters of complement. The mixtures were cooled and 10 c.c. of each of the mixtures treated as follows to remove the coagulable protein. The mixture was heated to 80° C. in a water bath, one per cent acetic acid was added until the first appearance of an acid reaction to

litmus paper and the mixture then boiled for a few minutes and filtered. The coagulable protein was washed with enough water to finally make the volume of the filtrate 100 c.c. and the amino-acids estimated as before. The remaining parts of the three mixtures were incubated under toluol for 16 hours and at the end of that time the amount of amino-acids in the uncoagulable protein of 10 c.c. of each estimated as above. The table shows that a marked increase in the amino-acids of the hemolytic mixtures occurred upon incubation.

Mixtures	Cubic Centimeter of 1/20 NaOH in Formol Titration	
	Before Incubation	After Incubation
Mixture I. 15 c.c. of blood suspension 3.5 c.c. of amboceptor, 6.0 c.c. of complement	0.6 c.c.	1.25 c.c.
Mixture II. 15 c.c. of blood suspension, 9.5 c.c. of salt solution	0.4 c.c.	0.4 c.c.
Mixture III. 15 c.c. of salt solution, 3.5 c.c. of amboceptor, 6.0 c.c. of complement	0.4 c.c.	0.4 c.c.

Repetition of this experiment gave constantly an increase in amino-acids in the corpuscle amboceptor complement mixtures during incubation, thus showing that proteolysis occurred during the process of hemolysis. Although the demonstration of the occurrence of proteolysis during hemolysis is not proof that it is the cause of the phenomenon, still it suggests rather strongly that such is the case.

CONCLUSIONS.

The results of this investigation would indicate that hemolytic complement is a proteolytic ferment which is either formed in the liver or is dependent upon liver activity for its presence in the blood.